This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.

Designation: D8243 – 19

Standard Test Method for Determination of APS Reductase to Estimate Sulfate Reducing Bacterial Bioburdens in Water – Enzyme-Linked Immunosorbent Assay Method[1]

This standard is issued under the fixed designation D8243; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method provides a protocol for using enzyme-linked immunosorbent assay (ELISA) technology to test water samples for the enzyme adenosine 5'-phosphosulfate reductase (APSr) concentration.

1.1.1 APSr is present in all known sulfate reducing protists (SRP – sulfate reducing bacteria – SRB – and sulfate reducing archaea – SRA).

1.1.2 As reported in U.S. Patent 4,999,286, APS reductase concentration can be used as a surrogate parameter for estimating SRA bioburdens (Appendix X1 compares results from Test Methods D8243, D4412, and quantitative polymerase chain reaction – qPCR – testing).

1.2 This test method has been validated in tap water, oilfield produced water (salinities ranging from 100 g L^{-1} to 600 g L^{-1}), and fuel-associated water (commonly referred to as bottoms-water).

1.3 This test method detects APS reductase semi-quantitatively in the range of 0.001M to 0.1M – correlating to 10^2 SRP/mL to 10^6 SRP/mL.

1.3.1 As described in Appendix X2 test method sensitivity can be increased 10-fold to 100-fold. However, the precision statistics provided in X apply only to 10-mL specimens.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.* Some specific hazards statements are given in Section 9 on Hazards.

1.6 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1129 Terminology Relating to Water

D4412 Test Methods for Sulfate-Reducing Bacteria in Water and Water-Formed Deposits

D5847 Practice for Writing Quality Control Specifications for Standard Test Methods for Water Analysis

D6300 Practice for Determination of Precision and Bias Data for Use in Test Methods for Petroleum Products and Lubricants

D6499 Test Method for Immunological Measurement of Antigenic Protein in Hevea Natural Rubber (HNR) and its Products

D7464 Practice for Manual Sampling of Liquid Fuels, Associated Materials and Fuel System Components for Microbiological Testing

E691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method

E1601 Practice for Conducting an Interlaboratory Study to Evaluate the Performance of an Analytical Method

E1847 Practice for Statistical Analysis of Toxicity Tests Conducted Under ASTM Guidelines

E1914 Practice for Use of Terms Relating to the Development and Evaluation of Methods for Chemical Analysis (Withdrawn 2016)[3]

2.2 *Patents:*[4]

U.S. Patent 4,999,286 Sulfate Reducing Bacteria Determination and Control, March 12, 1991

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[1] This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

[4] Available from United States Patent and Trademark Office (USPTO), Madison Building, 600 Dulany Street, Alexandria, VA 22314, https://www.uspto.gov.

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3. Terminology

3.1 *Definitions:*

3.1.1 For definitions of terms used in this standard, refer to Terminology D1129.

3.1.2 *adenosine 5'-phosphosulfate reductase (APS reductase), n*—(EC 1.8.4.9 and CAS 9027-75-2) an enzyme present on all known sulfate reducing bacteria and archaea, catalyzing the two-electron reduction of the sulfate group in adenosine 5'-phosphosulfate (APS) to yield adenosine monophosphate and sulfite.

3.1.3 *categorical data, n*—variates that take on a limited number of distinct values. **E1847**

3.1.4 *chromogen, n*—a chemical or compound that reacts with an enzyme to produce a colored end-product, used to detect the presence of a substance of interest.

3.1.5 *detection limit, n*—the smallest net signal (or the derived property value, constituent mass fraction, etc.) obtained by a given measurement procedure, that can be distinguished from the background signal at a specified confidence level. **E1914**

3.1.6 *enzyme linked immunosorbent assay (ELISA), n*—an immunological test method to quantify antigen or antibody levels using an enzyme as the detection mechanism. **D6499**

3.2 *Symbols:*

APSr—APS reductase
BDL—below detection limit
GHS—Globally Harmonized System of Classification and Labeling of Chemicals
SRA—sulfate reducing archaea
SRB—sulfate reducing bacteria
SRP—sulfate reducing protists

4. Summary of Test Method

4.1 A 10-mL water specimen is filtered through a diatomaceous earth filter (8.3) that captures microbial cells. The filtrate is discarded.

4.2 The wash bottle's cap (8.5) is replaced with the filter cap from 4.1. The bottle is inverted and the 2 mL of wash solution is filtered through the filter medium to eliminate interferences.

4.3 The filter cap is removed from the wash bottle and placed onto the bottle containing the rehydrated lysing reagent (8.2). The filter cake is dispersed from the cap into the lysing reagent and allowed to stand for 2 min.

4.4 The filter cap is replaced with the final filter cap (8.7) and the lysed solution is filtered into the immunoreagent vial (8.9). The filtrate-immunoreagent mixture is allowed to stand for 2 min.

4.5 The lysate-immunoreagent mixture is dispensed into the funnel device (8.8).

4.6 After all of the fluid has passed through the funnel device's filter membrane, the contents of the chromogen reagent dropper vial (8.5) are dispensed onto the funnel device's filter membrane. A laboratory-timer (7.1), set to the appropriate time (15 min at 18 ± 2°C) is started.

4.7 At the end of the color-development period (that is, 15 min at 18 ± 2°C), compare and match the color of the funnel device's filter membrane with the colors on the QuickChek[5] color card (8.6).

4.8 Report SRB/mL based on the color match observed in 4.7.

5. Significance and Use

5.1 Sulfate reducing archaea and bacteria are known to contribute to microbiologically influenced corrosion.

5.2 Sulfate-reducing bacteria are widely distributed in marine and fresh water muds which, in consequence, frequently are laden with the hydrogen sulfide produced by these organisms during dissimilatory sulfate reduction.

5.3 Traditional, culture-dependent methods such as those described in Test Methods D4412, prescribe incubation periods of as long as 21 days before assigning a below detection limit (BDL) score to a specimen. Moreover, it is well known that not all SRP will proliferate in the nutrient media specified in Test Methods D4412.

5.4 This test method uses ELISA technology to provide semi-quantitative, culture-independent, SRP bioburden test results in less than 30 min.

5.4.1 Because all the reagents and supplies used are non-hazardous and prepackaged for single test use, this test method does not require any apparatus other than a laboratory timer. Consequently, it can be performed at or near the point of sample collection.

5.4.2 The opportunity to minimize the delay between sample collection, testing, and results availability translates into timely use of the data to drive preventive and corrective SRB control measures.

6. Interferences

6.1 SRP population densities $>10^6$ cells/mL can cause an overload effect that can cause the population density to be underestimated.

7. Apparatus

7.1 Timer, laboratory

8. Reagents and Materials

8.1 Bottle, dropper, containing 2-mL lysing reagent rehydration fluid.[6]

8.2 Bottle, lysing reagent, proprietary, freeze-dried.

[5] QuickChek is a trademark of Modern Water, Inc, New Castle, DE, https://www.modernwater.com, and is the sole source of supply known to the committee at this time. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

[6] The sole source of supply of the proprietary chromogen liquid, final filter, immunoreagent, lysing reagent, rehydrating fluid, sample bottle with filter cap, test membrane device, and wash solution is Modern Water, Inc, New Castle, DE, https://www.modernwater.com. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

8.3 Bottle, sample, with proprietary filter cap, 10 mL.

8.4 Bottle, wash, containing: wash buffer, 2 mL, proprietary.

8.5 Chromogen, proprietary, in dropper vial.

8.6 Color card, QuickChek.

8.7 Filter, final, dispensing cap, proprietary.

8.8 Funnel device, proprietary.

8.9 Immunoreagent, proprietary, in vial.

8.10 Wash reagent, final, in dropper vial.

9. Hazards

9.1 Hydrogen sulfide can accumulate in any air space in sample containers filled with SRB-contaminated water. Hydrogen sulfide is flammable (GHS02), corrosive (GHS05), and toxic (GHS06). Appropriate precautions shall be taken when handling samples likely to contain detectable SRB populations.

10. Sampling, Test Specimens, and Test Units

10.1 Samples shall be collected and handled in accordance with guidance provided in Practice D7464.

10.1.1 Because microbes continue to be metabolically active in collected samples, microbiological parameters are perishable.

10.1.2 Optimally, samples shall be tested within 4 h after collection.

10.1.3 Acceptable test results can be obtained if testing is started within 24 h on samples that have been either kept on ice or refrigerated since collection.

10.1.4 As the delay between sampling and testing increases beyond 24 h the relationship between test results and SRP bioburdens in the systems from which samples were collected becomes increasingly tenuous.

10.2 The standard specimen for this test method is 10 mL.

10.3 The sample unit size is not critical, but a minimum unit size of 100 mL is recommended for replicate testing.

11. Calibration and Standardization

11.1 No calibration or standardization is needed for this test method. However, if a user is concerned about obtaining false negative results, reagent response can be tested in accordance with the protocol provided in Appendix X2.

12. Conditioning

12.1 Enzyme reaction rates are temperature dependent. Therefore, all samples and reagents shall be permitted to equilibrate to ambient temperature before testing. As explained in 13.7.3, the time allowed for ELISA reaction color development is temperature dependent.

13. Procedure

13.1 *Rehydrate Lysing Reagent:*

13.1.1 Remove caps from rehydrating solution dropper bottle (8.1) and lysing reagent bottle (8.2).

13.1.2 Invert rehydrating solution dropper bottle (8.1) over lysing reagent bottle (8.2) and squeeze the latter to dispense rehydrating solution into lysing reagent bottle.

13.1.3 Replace lysing reagent bottle cap, and gently swirl bottle for 15 sec to facilitate reagent rehydration.

13.2 *Collect Specimen:*

13.2.1 Shake sample vigorously for 30 sec to ensure that it is mixed properly.

13.2.2 Remove dropper cap from sample bottle (8.3).

13.2.3 Dispense 10 mL of sample into sample bottle (8.3) and replace dropper cap.

13.2.4 Invert sample bottle (13.2.3) and shake for 15 sec to disperse filter cake.

13.2.5 Keeping sample bottle (13.2.4) inverted, wait 15 sec to allow filter cake to settle into dropper tip.

13.2.6 Apply steady pressure to squeeze sample bottle (13.2.5) to filter sample.

13.2.6.1 Dispense filtrate into a waste container.

13.2.6.2 Maintain pressure on sample bottle until the entire 10-mL specimen has been filtered.

NOTE 1—While performing the filtration step, do not release pressure. Doing so will create a partial vacuum in the sample bottle. This will permit air to enter the bottle, causing the filter cake to re-disperse into the specimen and potentially cause SRP to be dispensed with the filtrate.

NOTE 2—A vacuum device may be used to facilitate filtration. However, the test results used to obtain the precision statistics provided for this test method were based on manual squeezing in accordance with 13.2.6.2.

13.3 *Wash Specimen:*

13.3.1 Remove cap from wash buffer bottle (8.4).

13.3.2 Remove filter cap from sample bottle (13.2.6).

13.3.3 Place cap (13.3.2) onto wash buffer (8.4).

13.3.4 Invert wash buffer bottle (13.3.3) and shake for 15 sec to disperse filter cake.

13.3.5 Keeping wash buffer bottle (13.3.4) inverted, wait 15 sec to allow filter cake to settle into dropper tip.

13.3.6 Apply steady pressure to squeeze wash buffer bottle (13.3.5) to filter sample.

13.3.7 Dispense filtrate into a waste container.

13.3.8 Maintain pressure on sample bottle until the entire 2-mL wash buffer has been filtered.

13.4 *Lyse Cells:*

13.4.1 Remove cap from lysing reagent bottle (13.1.3).

13.4.2 Remove dropper tip from wash buffer bottle (13.3.8) and place it onto lysing reagent bottle (13.4.1).

13.4.3 Flick the side of the lysing bottle/filter cap firmly with your index finger.

13.4.3.1 If the filter cake does not become dislodged, invert the bottle and flick it firmly again.

13.4.4 After the filter cake is dislodged, strike the base on a flat surface to make the filter cake fall into the lysing fluid.

13.4.5 If any of the cake remains in the filter cap, tip the bottle upside down and allow the fluid to run into the filter cap.

NOTE 3—When the fluid will go all the way to the plug in the cap, all the filter cake has been transferred.

13.4.6 Swirl lysing bottle (13.4.5) for 15 sec to mix the filter cake and lysing reagent well.

NOTE 4—Be careful not to mix so aggressively as to cause the filter cake dispersion to foam.

13.4.7 Remove filter cap and let lysing vial stand for 2 min.

13.4.8 Snap final filter dropper top (8.7) into place on lysing bottle (13.4.7).

13.5 *React Analyte with Immunoreagent:*

13.5.1 Remove the rubber cap from the glass immunoreagent vial (8.9).

13.5.2 Invert the lysing vial (13.4.8) over the immunoreagent vial (13.5.1) and squeeze the liquid from the lysing bottle into the immunoreagent vial.

NOTE 5—Between 10 and 20 drops of fluid should flow through the final filter into the immunoreagent vial.

13.5.3 Stop squeezing the lysing bottle when all the liquid is expelled, and foam appears at the filter tip.

13.5.4 Mix the liquid in the immunoreagent vial gently and let it incubate for two minutes.

NOTE 6—During this incubation period, the antibody-coated particles bind with the APS reductase in solution.

NOTE 7—The prescribed 2 min is nominal. Incubation intervals between 2 min and 4 min do not affect test results.

13.6 *Concentrate Antigen-Antibody Complexed Particles:*

13.6.1 Decant the contents of the immunoreagent vial (13.5.4) onto the funnel device's membrane (8.8).

13.6.2 Allow all fluid to be drawn through the funnel device's membrane.

NOTE 8—In the funnel device (8.8) the filter membrane rests on an absorbent bed. The absorbent draws the fluid through the membrane. The time required for all of the fluid to drain through the funnel device's membrane can vary from 2 min to 5 min.

13.6.3 Remove yellow cap from final wash reagent vial (8.10) and squeeze vial to dispense wash reagent onto membrane.

13.6.4 Allow all fluid to be drawn through the funnel device's membrane.

13.7 *Color Development:*

13.7.1 Remove blue cap from chromogen vial (8.5).

13.7.2 Invert vial and squeeze its sides to dispense chromogen onto funnel device's membrane (13.6.4).

13.7.3 Set lab timer to appropriate time in accordance with Table 1.

13.7.4 Allow membrane color to develop for period set in 13.7.3.

13.7.4.1 Do not perform this test method if the ambient temperature is <16°C or >38°C.

13.8 *Observe Color:*

13.8.1 Place QuickChek color card (8.6) near funnel device membrane as shown in Fig. 1.

13.8.2 Align color card (8.6) with membrane to match membrane's color with appropriate color on card.

13.8.3 Record SRP/mL based on color match.

TABLE 1 Relationship Between Ambient Temperature and Chromogen Color Development Time

Temperature °C	Color Development Time (Min)
16 to 20	15
>20 to 26	10
>26 to 32	8
>32 to 38	6

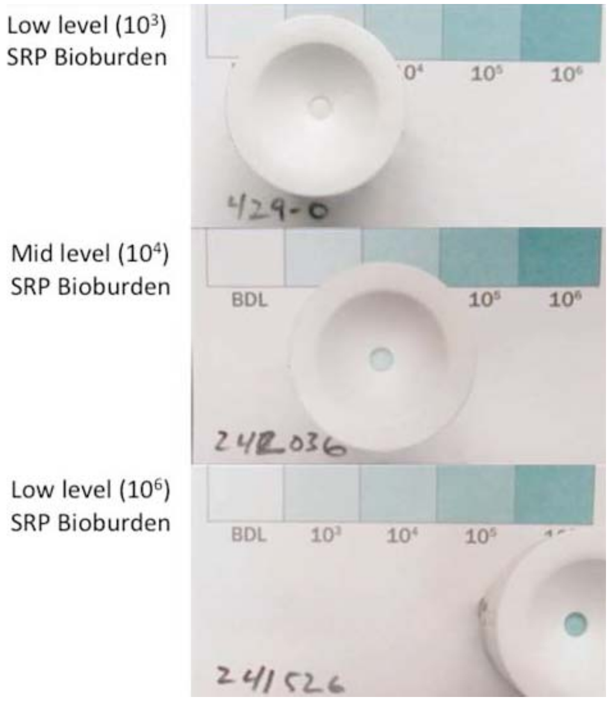

FIG. 1 QuickChek Color Card Next to Filter Device Membrane to Facilitate SRP Bioburden Scoring

14. Calculation or Interpretation of Results

14.1 For 10-mL specimens, no calculations are required. The reported SRP/mL is read directly from the QuickChek color card (8.6) in accordance with 13.8.3.

14.2 *Interpretation:*

14.2.1 SRP upper control limits can vary, depending on the system from which samples are collected.

14.2.2 In many industrial applications, any SRP bioburden that is greater than BDL triggers follow-up action.

14.2.3 Some applications set the upper control limit at $<10^3$ SRP/mL.

15. Reporting

15.1 Recognizing that SRP/mL can range from $<10^2$ SRP/mL to $>10^6$ SRP/mL, Log_{10} transformation facilitates data entry and process control plotting.

15.1.1 To transform test results, compute Log_{10} of the observed cells/mL as in Eq 1:

$$Log_{10}(10^3 \quad SRP/mL) = 3 Log_{10} SRP/mL \qquad (1)$$

15.1.2 After Log_{10} transformation, test results are reported as <2 (that is, BDL), 2, 3, 4, 5, 6 or >6.

16. Precision and Bias

16.1 *Precision:*

16.1.1 Test results are reported as categorical data (that is, whole numbers only). Consequently, neither Practice E691, nor Practice E1601 apply to this test method. Because results are categorical, statistical analysis assuming continuous function results overestimates variability. However, an interlaboratory study (ILS) was performed to estimate intermediate precision.

16.1.1.1 The preliminary ILS details are provided in Appendix X4. Briefly, three analysts each ran the test method in duplicate on fuel-associated bottoms-water and phosphate buffered saline that had been spiked with three different SRB bioburdens (~10^6 SRB/mL, ~10^4 SRB/mL, and ~10^3 SRB/mL). Unspiked bottoms-water and phosphate buffered saline served as BDL controls.

16.1.1.2 The preliminary repeatability limit:

$$r = 1.91\ \mathrm{Log}_{10}\ \mathrm{SRP/mL} \quad (2)$$

16.1.1.3 The intermediate, preliminary reproducibility limit:

$$R = 9.91\ \mathrm{Log}_{10}\ \mathrm{SRP/mL} \quad (3)$$

16.2 *Bias*—No information can be presented on the bias of the procedure in this test method for measuring SRP/mL because no material having an accepted reference value is available.

17. Quality Control (QC)

17.1 In accordance with Practice D5847, the following quality control guidance is provided.

17.1.1 *Initial Demonstration of Laboratory Capability*—Laboratories performing this test method for the first time shall prepare a dilution curve in accordance with instructions provided in Appendix X3. Each individual analyst who will be performing the test shall perform duplicate tests at ≥3 SRP/mL bioburdens. The repeatability agreement score shall be no less than 90 %, agreement among multiple analysts shall be no less than 80 %, and agreement between novice and expert analysts shall be no less than 80 %.

17.1.2 *Batch QC*—Replicate tests of a single sample shall not vary by >1 categorical value (for example, 3 ± 1 SRP/mL).

17.1.3 For the purposes of precision evaluation, no fewer than duplicate specimens shall be tested.

17.1.4 *Method Blank*—Background SRP/mL shall be determined by performing this test method on a sterile aqueous specimen.

NOTE 9—For the method blank, it is preferable, although not mandatory, to use a fluid similar to the test sample matrix. For example, if test samples will be oilfield produced water with salinity of 5000 mg/L, use sterile synthetic seawater, with salinity adjusted to 5000 mg/L, as the method blank.

17.1.5 The background SRP/mL shall not exceed $2\mathrm{Log}_{10}$.

17.1.6 If the background SRP/mL does exceed $2\mathrm{Log}_{10}$, replace all supplies from a given lot of test kit consumables with supplies from a different production lot.

17.1.7 *Laboratory Control Sample (LCS)*—Calibration is achieved in accordance with Appendix X3.

17.1.8 *Matrix Spike (MS)*—Follow guidance provided in Appendix X2 to test for interferences or other matrix effects.

17.1.9 *Independent Reference Sample (IRS)*—There is no known independent reference sample other than the 0.1M APSR reagent described in Appendix X3. Users who are aware of alternative reference standards are advised to provide this information to ASTM International Headquarters. All comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

18. Keywords

18.1 APS reductase; SRA; SRB; SRP; sulfate reducing archaea; sulfate reducing bacteria; sulfate reducing protists

APPENDIXES

(Nonmandatory Information)

X1. RELATIONSHIP BETWEEN APSR CONCENTRATION AND SRP/ML

X1.1 Field Testing – Oilfield Produced Water Samples

X1.1.1 Fifty oilfield produced water samples were tested by Test Method D8243, Test Methods D4412, and a quantitative polymerase chain reaction (qPCR) method.

X1.1.2 Table X1.1 compares the attribute scores for the three methods:

- 1 = negligible contamination – no corrective action required;
- 3 = moderate contamination – preventive action recommended; and
- 5 = heavy contamination – corrective action needed).

X1.1.3 Agreement between Test Methods D8243 and D4412, respectively, and qPCR was the same (66 %).

X1.1.4 The results of Test Methods D8243 and D4412 agreed for 80 % of all samples.

X1.1.5 For 82 % and 90 % of the samples, respectively, Test Method D8243 results were either equal to or more conservative than Test Methods D4412 or qPCR results.

TABLE X1.1 Test Method Comparison – Test Method D8243, Test Methods D4412, and qPCR Attribute Score Agreement

Parameters	D8243 = D4412	D8243 = dsrB[A]	D4412 = dsrB	D8243 ≥ D4412	D8243 ≥ dsrB	D4412 ≥ dsrB
Total	40	33	33	45	41	4182
%	80	66	66	90	82	

[A] dsrB is the target gene for quantifying SRP by quantitative polymerase chain reaction (qPCR) testing.

X2. INCREASING TEST METHOD SENSITIVITY

X2.1 Test Method D8243 sensitivity can be increased 10-fold using supplies and reagents listed in 8.1 through 8.10 so that the limit of detection is decreased to 10 SRP/mL (1Log$_{10}$ SRP/mL).

X2.2 After completing steps 13.1 through 13.2.6, keep the sample bottle (13.2.5) inverted.

X2.3 Remove the sample bottle's base.

X2.3.1 Dip either a scalpel, box-cutter blade, or scissors into either ethanol or methanol (≥70 % alcohol by vol) and let alcohol evaporate.

NOTE X2.1—If working in a microbiology laboratory and a laboratory burned is available, pass implement through flame to ignite residual alcohol. *Do not use flame in the vicinity of flammable materials.*

X2.3.2 Use disinfected scalpel, box-cutter blade, or scissors to cut off sample bottle base. Sample bottle will become a funnel.

X2.4 Filter Additional Sample

NOTE X2.2—Vacuum filtration is needed to draw additional sample through the sample bottle filter. The recommended apparatus in a 250-mL polypropylene filtration flask fitted with a #6½, one-holed stopper. Sample bottle, filter cap may be positioned into stopper hole either before or after performing 13.2.6 on 10-mL specimen.

X2.4.1 Shake original sample from which first 10-mL specimen (13.2.3) was decanted.

X2.4.2 Dispense an additional 10-mL specimen into sample bottle (X2.3.2).

X2.4.3 Wait until approximately 80 % of the fluid in the sample bottle (X2.3.2) has been filtered.

X2.4.4 Repeat X2.4.1 through X2.4.3 until a total (including first 10-mL portion – 13.2.3) of 100 mL has been filtered.

NOTE X2.3—The total sample volume that can be filtered will decrease as its particle load increases.

NOTE X2.4—Although filtration often becomes difficult as the total sample volume approaches 100 mL, additional 10 mL volumes can be added and filtered until the time required to filter the fluid in sample bottle in greater than 5 min.

X2.4.5 Maintain vacuum until all sample fluid has been filtered.

X2.4.6 Record the total sample volume filtered.

X2.5 Complete 13.4 through 13.8.

X2.6 Compute Concentration Factor and Correct Test Result

X2.6.1 Divide total volume filtered (X2.4.6) by 10 mL.

$$C = V_s \text{ mL} \div 10 \text{ mL} \tag{X2.1}$$

where:

C = the concentration factor, and
V_s = the total sample volume.

X2.6.2 Divide the test result (13.8.3) by C to obtain the corrected SRP/mL.

X2.6.3 If desired, convert the corrected SRP/mL to Log$_{10}$ SRP/mL.

X3. TEST METHOD D8243 CALIBRATION

X3.1 This test method is a semi-quantitative protocol that yields categorical data. Users can use either a two-sample response test or an APSr calibration curve for quality control purposes.

X3.2 Response Test

X3.2.1 *Reagents:*

X3.2.1.1 Sterile, distilled or deionized water, 10 mL.

X3.2.1.2 Positive control reconstitution fluid, proprietary.

X3.2.1.3 Positive control reagent, freeze dried, in vial.

X3.2.2 *Negative Control:*

X3.2.2.1 Perform this test method on 10-mL sterile specimen (X3.2.1.1). Log$_{10}$ SRP/mL should be ≤2.

- If Log$_{10}$ SRP/mL >2, repeat this test method with a second sterile specimen.
- If Log$_{10}$ SRP/mL > 2 for second specimen, discard supplies from test kit production lot used to perform negative control test.

X3.2.3 *Positive Control:*

X3.2.3.1 Remove caps from positive control reconstitution fluid (X3.2.1.2) and positive control reagent vial (X3.2.1.3).

X3.2.3.2 Dispense positive control reconstitution fluid (X3.2.1.2) into vial containing positive control reagent (X3.2.1.3) and mix gently to rehydrate positive control reagent.

X3.2.3.3 Decant rehydrated positive control reagent into immunoreagent vial (8.9) and complete protocol in accordance with 13.5.4 through 13.8. Log$_{10}$ SRP/mL should be ≥5.

- If Log$_{10}$ SRP/mL <5, repeat this test method with a second sterile specimen.
- If Log$_{10}$ SRP/mL <5 for second specimen, discard supplies from test kit production lot used to perform negative control test.

X3.3 Calibration Curve

X3.3.1 *Reagents:*

X3.3.1.1 *Adenosine 5'-Phosphosulfate Reductase, 1.0 M.*

X3.3.1.2 *Adenosine 5'-Phosphosulfate Reductase, 0.1 M*—Mix 10 mL of adenosine 5'-phosphosulfate reductase, 1.0 M (X3.3.1.1) with 90 mL phosphate buffered saline (X3.3.1.5).

X3.3.1.3 *Adenosine 5'-Phosphosulfate Reductase, 0.01 M*—Mix 10 mL of adenosine 5'-phosphosulfate reductase, 0.1 M (X3.3.1.2) with 90 mL phosphate buffered saline (X3.3.1.5).

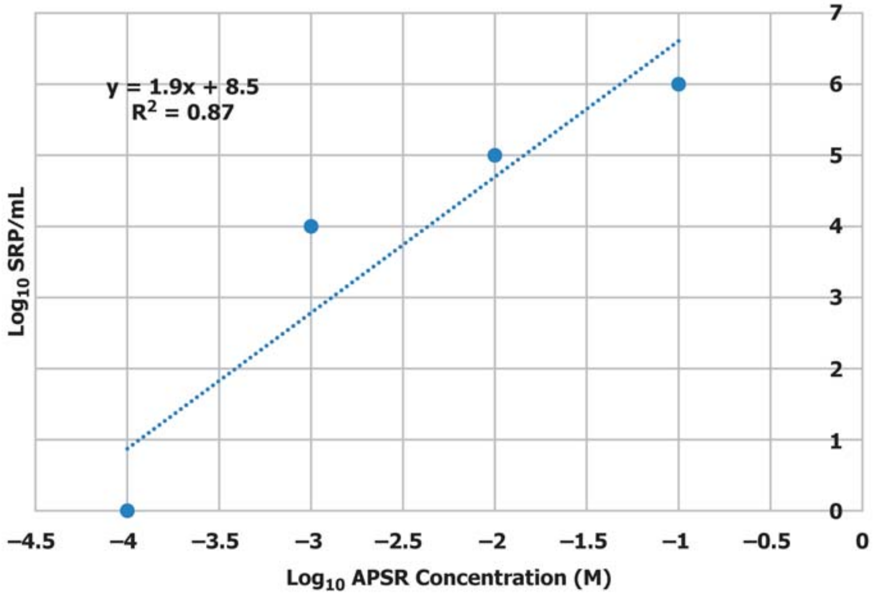

FIG. X3.1 Calibration Curve: Log_{10} Adenosine 5'-Phosphosulfate Reductase (M) versus Test Method D8243 Results (Log_{10} SRP/mL)

TABLE X3.1 Calibration Curve Data: Log_{10} Adenosine 5'-Phosphosulfate Reductase (M) versus Test Method D8243 Results (Log_{10} SRP/mL)

Log_{10} [APSR] (M)	Log_{10} SRP/mL
–1	6
–2	5.0
–3	4
–4	0
Slope	1.90
R^2	0.87

X3.3.1.4 *Adenosine 5'-Phosphosulfate Reductase, 0.001 M*—Mix 10 mL of adenosine 5'-phosphosulfate reductase, 0.01 M (X3.3.1.3) with 90 mL phosphate buffered saline (X3.3.1.5).

X3.3.1.5 *Phosphate Buffered Saline*—Dissolve 8 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 1.44 g disodium phosphate, dihydrate ($Na_2HPO_4 . 2H_2O$), 0.24 g dibasic potassium phosphate (KH_2PO_4), in distilled or deionized water. Use hydrochloric acid (HCl) to adjust pH to 7.4. Add distilled or deionized water to 1 L. To sterilize, autoclave in accordance with standard microbiological laboratory practice.

NOTE X3.1—Commercially prepared, sterile phosphate buffered saline is available from scientific supply distributors.

X3.3.2 *Protocol:*

X3.3.2.1 Perform this test method on 0.1 M, 0.01 M, 0.001 M adenosine 5'-phosphosulfate reductase solutions (X3.3.1.2 through X3.3.1.4) and on sterile phosphate buffered saline (X3.3.1.5).

X3.3.2.2 Prepare a table and plot of Log_{10} SRP/mL versus Log_{10} adenosine 5'-phosphosulfate reductase molar concentration as illustrated in Table X3.1 and Fig. X3.1.

X3.3.2.3 Compute slope and correlation coefficient of Log_{10} SRP/mL versus Log_{10} adenosine 5'-phosphosulfate reductase molar concentration.

- Correlation coefficient R^2 should be ≥0.85.

X4. PRELIMINARY INTERLABORATORY [RUGGEDNESS] STUDY

X4.1 This Ruggedness Study (ILS) was conducted to establish a preliminary repeatability statement for Test Method D8243.

X4.2 Participants

X4.2.1 The following laboratory participated in this study—Modern Water, Inc., New Castle, DE.

X4.2.2 Participating analysts were: Jim Gill, Sejal Patel, and Katie Powell. All participants were Modern Water employees.

X4.3 Description of Samples

X4.3.1 Sulfate reducing bacteria (SRB) cell suspension (SRBS) was cultivated in accordance with NACE TM 0194 by Dr. John Kilbane of Intertek Westport technology Center, Houston, TX. A 500-mL batch of SRB media was inoculated with fresh SRB isolated from an oilfield sample and incubated at 30°C for one week to obtain an SRB CFU/mL population density of 1.1×10^7. The SRBS was placed on ice and shipped to Modern Water by means of overnight delivery.

X4.3.2 Ultra-low-sulfur diesel (ULSD) fuel was provided by Fairville Products, Wilmington, DE.

X4.3.3 Phosphate buffered saline (PBS) pH 7.4, 0.1 M; was prepared from SIGMA; Pcode 1002467507. In accordance with manufacturer's instructions, one foil packet was dissolved in 1 L deionized water.

X4.3.4 SRBS was diluted into volumes of PBS to give the estimated SRB bioburdens listed Table X4.1.

X4.3.5 To simulate bottoms-water samples, 15 mL ULSD was dispensed into 24 50-mL polypropylene centrifuge tubes. Then, 15 mL of SRBS, or SRBS in PBS was added. Tubes were then shaken and permitted to phase-separate for ≥30 min before ILS began.

X4.3.6 For fuel-free specimens, 15 mL of SRB was dispensed into 50-mL centrifuge tubes.

X4.3.7 The test matrix is show in Table X4.2. Six specimens were prepared for each sample-type and bioburden. Each participant was provided with a sample rack with specimens numbered 1 through 16.

X4.4 ILS Instructions

X4.4.1 Analysts were instructed to perform the test in accordance with the steps provided in Section 13, and results were read and reported in accordance with Sections 13 and 14.

X4.5 Statistical Data Summary

X4.5.1 SRB test results are shown in Table X4.3.

X4.5.2 Two-way analysis of variance (ANOVA) statistics are provided in Table X4.4. The contribution of analyst to test method variability was not significant at the 95 % confidence level. As expected, the contribution of sample was significant. There was an interaction effect between the two factors. Based on visual examination of the data sets, it appeared that Analyst B's data set contributed significantly to the observed interaction effect. A second two-way ANOVA was computed. As seen in Table X4.5, when Analyst B's data set was removed from the matrix, the integration effect was no longer significant.

TABLE X4.1 Dilution of SRBS to Deliver Target SRB Bioburdens

Target Cells/mL	SRBS[A] (mL)	PBS (mL)	Dilution Designation
$\geq 10^6$	150	0	A
10^4	2.5	247.5	B
10^3	25	225	C
$<10^2$	25	225	D

[A] The SRBS volumes listed in each of the last three rows were taken from the previous dilution, not the original SRBS (that is, the $<10^2$ cells mL^{-1} suspension was prepared from 25 mL of dilution *C* into 225 mL PBS).

TABLE X4.2 Preliminary ILS Sample Matrix

Sample	Test Fluid	Target SRB (cells mL^{-1})
1	Bottoms-water	$<10^2$
2	Bottoms-water	$<10^2$
3	PBS	$<10^2$
4	PBS	$<10^2$
5	Bottoms-water	10^3
6	Bottoms-water	10^3
7	PBS	10^3
8	PBS	10^3
9	Bottoms-water	10^4
10	Bottoms-water	10^4
11	PBS	10^4
12	PBS	10^4
13	Bottoms-water	10^6
14	Bottoms-water	10^6
15	PBS	10^6
16	PBS	10^6

X4.5.3 Two-way ANOVA was computed for the sample type versus SRB-bioburden data. As shown in Table X4.6, the sample type did not affect the test results significantly at the 95 % confidence level. The only significant factor was SRB-bioburden.

X4.5.4 Practice D6300 D2PP software was used to compute preliminary repeatability and reproducibility coefficients.

X4.5.4.1 *Preliminary Repeatability*—The quantitative expression for the random error associated with the difference between two independent results obtained under repeatability conditions that would be exceeded with an approximate probability of 5 % (one case in 20 in the long run) in the normal and correct operation of this test method.

$$\text{Repeatability}, r = 1.91\ \text{Log}_{10}\ \text{SRP/mL} \quad (X4.1)$$

X4.5.4.2 *Intermediate, Preliminary Reproducibility*—The quantitative expression for the random error associated with the difference between two independent results obtained under reproducibility conditions that would be exceeded with an approximate probability of 5 % (one case in 20 in the long run) in the normal and correct operation of this test method, when testing is performed in a single laboratory by different analysts.

$$\text{Intermediate reproducibility}, R = 9.91\ \text{Log}_{10}\ \text{SRP/mL} \quad (X4.2)$$

TABLE X4.3 Preliminary ILS Test Results – Log_{10} SRB Cells/mL

Analyst	Sample							
	BW-0	BW-3	BW-4	BW-6	PBS-0	PBS-3	PBS-5	PBS-6
A	2	3	5	5	1	3	3.5	4.5
	1	2	3	5	1	3	5	4
B	0	2	3.5	3.5	5	2	2	3.5
	2	2	3.5	3.5	4	3	3	3.5
C	3	3	4	5	0	2	3	4.5
	3	2	3	5	0	0	5	4
Avg	1.8	23	3.7	4.5	1.8	2.2	3.6	4.0
s	1.2	0.5	0.8	0.8	2.1	1.2	1.2	0.4

TABLE X4.4 Two-Way ANOVA Summary – Analyst (3) × Sample (8)

Source of Variation	SS	df	MS	F	P-value	F crit
Analyst	0.95	2	0.47	0.88	0.43	3.40
Sample	47.37	7	6.77	12.61	1.10E-06	2.42
Interaction	38.05	14	2.72	5.07	2.59E-04	2.13
Within	12.88	24	0.54			
Total	99.24	47				

TABLE X4.5 Two-Way ANOVA Summary – Analyst (2) × Sample (8)

Source of Variation	SS	df	MS	F	P-value	F crit
Analyst	0.63	1	0.63	1.08	0.31	4.49
Sample	61.74	7	8.82	15	5.93E-06	2.66
Interaction	6.93	7	0.99	1.69	0.18	2.66
Within	9.38	16	0.59			
Total	78.68	31				

TABLE X4.6 Two-Way ANOVA Summary – SRB-Bioburden (4) × Fluid-Type (2)

Source of Variation	SS	df	MS	F	P-value	F crit
Analyst	47	3	15.51	11.96	9.99E-06	2.84
Sample	0.42	1	0.42	0.33	0.57	4.08
Interaction	0.43	3	0.14	0.11	0.95	2.84
Within	52	40	1.30			
Total	99	47				

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